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Anti-cancer effect of ursolic acid activates apoptosis through ROCK/PTEN mediated mitochondrial translocation of cofilin-1 in prostate cancer

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Abstract. Ursolic acid is a type of pentacyclic triterpene compound with multiple pharmacological activities including cancer resistance, protection from liver injury, antisepsis, anti-inflammation and antiviral activity. The present study aimed to investigate the anticancer effect of ursolic acid. Ursolic acid activates cell apoptosis and its pro-apoptotic mechanism remains to be fully elucidated. Cell Counting kit-8 assays, flow cytometric analysis and analysis of caspase-3 and caspase-9 activity were used to estimate the anticancer effect of ursolic acid on DU145 prostate cancer cells. The protein expression of cytochrome *c*, rho-associated protein kinase (ROCK), phosphatase and tensin homolog (PTEN) and cofilin-1 were examined using western blot analysis. In the present study, ursolic acid significantly suppressed cell growth and induced apoptosis, as well as increasing caspase-3 and caspase-9 activities of DU145 cells. Furthermore, cytoplasmic and mitochondrial cytochrome *c* protein expression was significantly activated and suppressed, respectively, by ursolic acid. Ursolic acid significantly suppressed the ROCK/PTEN signaling pathway and inhibited cofilin-1 protein expression in DU145 cells. The results of the present study indicate that the anticancer effect of ursolic acid activates cell apoptosis through ROCK/PTEN mediated mitochondrial translocation of cofilin-1 in prostate cancer.

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Introduction

Prostate carcinoma is common among elderly men and has a high incidence in Europe and America, being second in terms of cancer-associated mortality (1). In East Asia, the morbidity of prostate cancer is relatively low but has demonstrated a rising trend due to the aging population, dietary structure alterations and improvements in diagnosis (2). Prostate carcinoma is currently the focus of much research attention, and if identified early and treated correctly patient quality of life and treatment efficacy will be high (3).

According to previous studies, the use of Chinese herbs to treat tumors is achieving increasing recognition (4-6). Previous studies have reported that Traditional Chinese Medicine (TCM) has effects on androgen-dependent and -independent prostate carcinoma (7-9). At present, there are seldom resources of TCM for the treatment of prostate carcinoma and the results of clinical and experimental studies are indispensable for further data regarding TCM therapy for the treatment of this disease (10).

The natural compounds extracted by TCM have been widely used to treat a number of diseases (11). Researchers have studied structural modification and artificial synthesis using the natural structure as a template to investigate the structure of these compounds (12). At present, numerous natural compounds utilized as medicines have demonstrated multiple chemical properties (13). These natural medicines are a homologous series of natural products composed from important sources of natural compounds whose structures are combined together for modern drug development (14). For example, pentacyclic triterpene compounds are a type of plant secondary metabolite with clinical antitumor research and development value, and the most in-depth studies concerning these compounds have been performed on lupane, oleanolic acid and ursolic acid (15). Ursolic acid is pentacyclic triterpene compound that is widespread in nature, and is present in the leaves and fruits of *Ericaceae* bearberry, leaves of *Scrophulariaceae paulownia tomentosa* and *Oleaceae* privet (16). Ursolic acid has extensive biological activity, including cancer resistance, protection from liver injury, antisepsis, anti-inflammation and antiviral activity (13,17).

It has previously been reported that only dephosphorylated cofilin can translocate into mitochondria to induce apoptosis, while phosphorylation inhibits mitochondrial translocation of cofilin and, thus, apoptosis (18). Phosphatase and tensin homolog (PTEN) is the substrate of rho-associated protein kinase 1 (ROCK1) kinase, which is involved in regulating cell survival and cell death. Substantial evidence indicates that the activation of Ras homolog gene family, member A (RhoA)/ROCK1 can increase PTEN activity, thus, inhibiting the activation of Akt. Furthermore, ROCK1 can lead to the dephosphorylation of cofilin by activating protein phosphatase 1/2A, inducing cofilin mitochondrial translocation and leading to mitochondrial damage (19). In addition, ROCK1 can induce the translocation of dynamin-related protein 1 (Drp1) into mitochondria by regulating the phosphorylation state of Drp1, resulting in remodelling of the morphology of mitochondria (20).

In the present study, evidence was provided that indicates that the anticancer effect of ursolic acid may activate apoptosis of prostate cancer cells via ROCK/PTEN-mediated mitochondrial translocation of cofilin-1.

Materials and methods

Reagents. RPMI-1640 medium and fetal bovine serum were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Ursolic acid (with a purity of 90%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and its chemical structure is indicated in Fig. 1. Cell Counting kit (CCK)-8 and bicinchoninic acid protein assays were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit was obtained from BestBio (Shanghai, China).

Cell culture. DU145 human prostate cancer cells were obtained from the Affiliated Hospital of Qingdao University (Qingdao, China), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 g/ml streptomycin and 100 U/ml penicillin) at 37˚C in an atmosphere of 5% CO_2.

Analysis of cell growth. DU145 cells were seeded into 96-well plates at a density of $1x10^4$ cells/well and cultured with complete medium containing various concentrations of ursolic acid (0, 10, 20, 40 and 80 μM) for 24, 48 and 72 h. Following ursolic acid treatment, CCK-8 reagent was added to the cells and incubated for 4 h at 37˚C in an atmosphere of 5% CO_2. Subsequently, the absorbance of each well was detected at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow cytometric analysis for cell apoptosis. DU145 cells were seeded into 6-well plates at a density of $1\text{-}2x10^6$ cells/well and cultured with complete medium containing various concentrations of ursolic acid (0, 10, 20 and 40 μM) for 48 h. DU145 cells were washed with cold phosphate-buffered saline twice and resuspended using 500 μl of binding buffer. Following resuspension, 5 μl of Annexin V-FITC and 10 μl of PI were added and incubated for 10 min at 4˚C in the dark. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

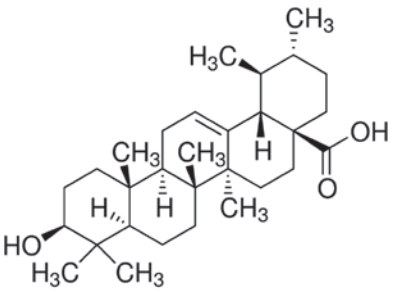

Figure 1. Chemical structure of ursolic acid.

Analysis of caspase-3 and caspase-9 activity. DU145 cells were seeded into 6-well plates at a density of $1\text{-}2x10^6$ cells/well and cultured with complete medium containing various concentrations of ursolic acid (0, 10, 20 and 40 μM) for 48 h. Cells were collected and the total protein concentration was determined using the bicinchoninic acid protein assay. Proteins were blended with 100 μl of Caspase-Glo 3 or Caspase-Glo 9 reagent (Promega Corporation, Madison, WI, USA) and incubated for 2 h at room temperature. Luciferase activity was measured using a TD 20/20 luminometer (Promega Corporation).

Western blot analysis. DU145 cells were seeded into 6-well plates at a density of $1\text{-}2x10^6$ cells/well and cultured with complete medium containing various concentrations of ursolic acid (0, 10, 20 and 40 μM) for 48 h. DU145 cells were prepared using a ProteoJET cytoplasmic protein extraction kit (Fermentas; Thermo Fisher Scientific, Inc.) or a Mitochondrial Fractionation kit (Active Motif, Shanghai, China). The mixed liquor was collected to determine the total protein concentration using the bicinchoninic acid protein assay. Protein (50 μg) was loaded onto 10-12% SDS-PAGE gels for electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membranes (0.22 mm) and blocked with Tris-buffered saline containing 5% non-fat milk for 2 h at room temperature. Subsequently, PVDF membranes were incubated with primary antibodies against the following: Cytochrome *c* (dilution, 1:2,000; #BBA2469; BestBio), ROCK (dilution, 1:1,000; #BBA5547; BestBio), PTEN (dilution, 1:1,000; #BBA5274; BestBio) and cofilin-1 (dilution, 1:2,000; #BBA2205; BestBio) overnight at 4˚C. PVDF membranes were subsequently incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (dilution, 1:1,000; #BB-2201-1; BestBio) at room temperature for 2 h and visualized by enhanced chemiluminescence. Protein expression was quantified using the ChemiDoc™ XRS system (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are expressed as the mean ± standard deviation. Analysis of variance was performed followed by the Student-Newman-Keuls method for pairwise comparison. SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA) was used to perform all statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

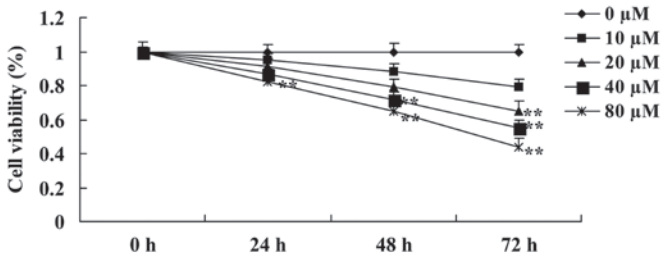

Figure 2. Anticancer effect of ursolic acid treatment on growth in prostate cancer cells. **P<0.01 compared with 0 μM ursolic acid group.

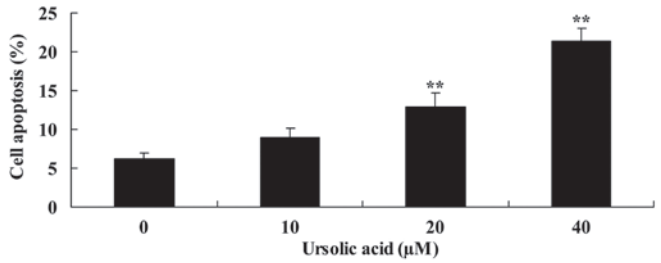

Figure 3. Anticancer effect of ursolic acid treatment on apoptosis in prostate cancer cells. **P<0.01 compared with 0 μM ursolic acid group.

Results

Anticancer effect of ursolic acid treatment on cell growth in prostate cancer cells. Initially, the present study investigated the anticancer effect of ursolic acid treatment on the growth of DU145 cells. The results of the present study revealed the anticancer effect of ursolic acid treatment was able to reduce the growth of DU145 cells in a time- and dose-dependent manner (Fig. 2). Notably, when cells were treated with 20 μM of ursolic acid for 72 h, 40 μM of ursolic acid for 48 and 72 h or 80 μM of ursolic acid for 24, 48 and 72 h, the growth of DU145 cells was significantly decreased compared with treatment with 0 μM of ursolic acid (Fig. 2).

Anticancer effect of ursolic acid treatment on cell apoptosis in prostate cancer cells. Subsequently, the present study investigated the anticancer effect of ursolic acid treatment on apoptosis of DU145 cells. As shown in Fig. 3, treatment with ursolic acid (20 and 40 μM) significantly increased apoptosis of DU145 cells at 48 h in a dose-dependent manner, compared with treatment with 0 μM of ursolic acid (Fig. 3).

Anticancer effect of ursolic acid treatment on caspase-3 and caspase-9 activity in prostate cancer cells. Subsequently, the present study investigated the anticancer effect of ursolic acid treatment on caspase-3 and caspase-9 activity of DU145 cells. Compared with treatment with 0 μM of ursolic acid, treatment with ursolic acid (20 and 40 μM) significantly induced caspase-3 and caspase-9 activity of DU145 cells at 48 h in a dose-dependent manner (Fig. 4).

Anticancer effect of ursolic acid treatment on cytochrome c protein expression in prostate cancer cells. To investigate the underlying anticancer mechanism of ursolic acid on prostate cancer cells, cytochrome *c* protein expression in the cytoplasm and mitochondria was measured using western blotting. The results of the present study indicated that treatment with ≥20 μM ursolic acid for 48 h significantly activated cytochrome *c* protein expression in the cytoplasm of DU145 cells and suppressed cytochrome *c* protein expression in the mitochondria of DU145 cells, compared with treatment with 0 μM of ursolic acid (Fig. 5).

Anticancer effect of ursolic acid treatment on ROCK protein expression in prostate cancer cells. To observe the underlying mechanism of ursolic acid action on prostate cancer cells, ROCK protein expression was measured using western blotting. The results of the present study revealed that treatment with 20 and 40 μM ursolic acid for 48 h significantly suppressed

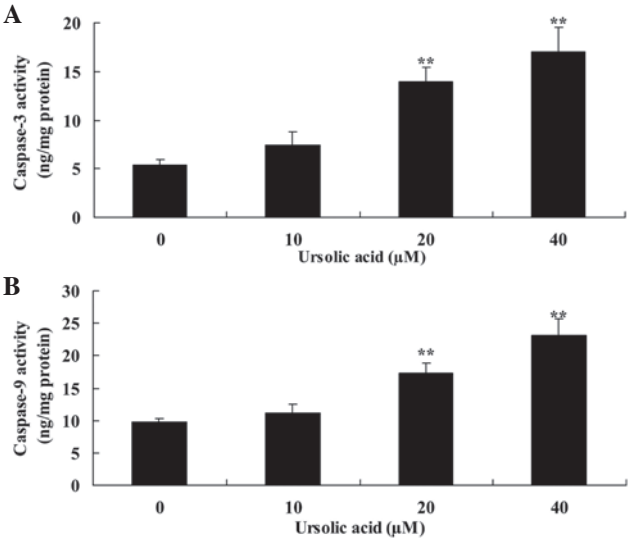

Figure 4. Anticancer effect of ursolic acid treatment on (A) caspase-3 and (B) caspase-9 activity in prostate cancer cells. **P<0.01 compared with 0 μM ursolic acid group.

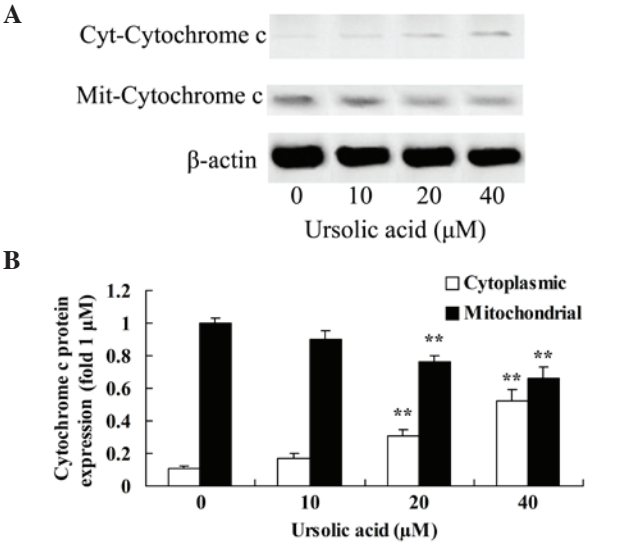

Figure 5. Anticancer effect of ursolic acid treatment on cytochrome *c* protein expression in prostate cancer cells. Anticancer effect of ursolic acid treatment on cytochrome *c* protein expression assessed (A) by western blotting assays and (B) quantification of cytochrome *c* protein expression in prostate cancer cells. **P<0.01 compared with 0 μM ursolic acid group.

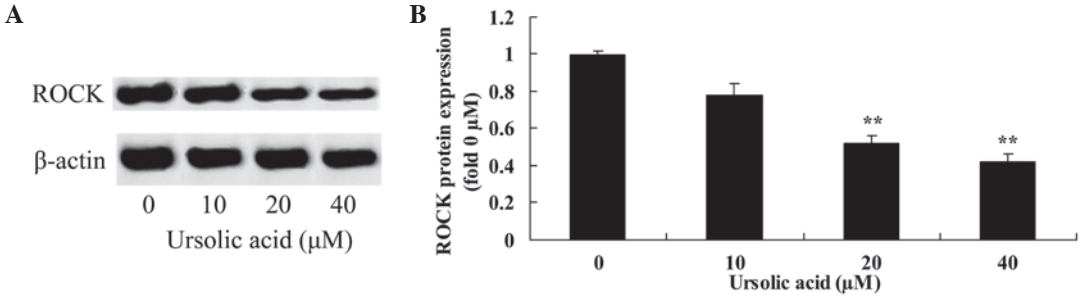

Figure 6. Anticancer effect of ursolic acid treatment on ROCK protein expression in prostate cancer. Anticancer effect of ursolic acid treatment on ROCK protein expression assessed (A) by western blotting assays and (B) quantification of ROCK protein expression in prostate cancer cells. **P<0.01 compared with 0 μM ursolic acid group. ROCK, rho-associated protein kinase.

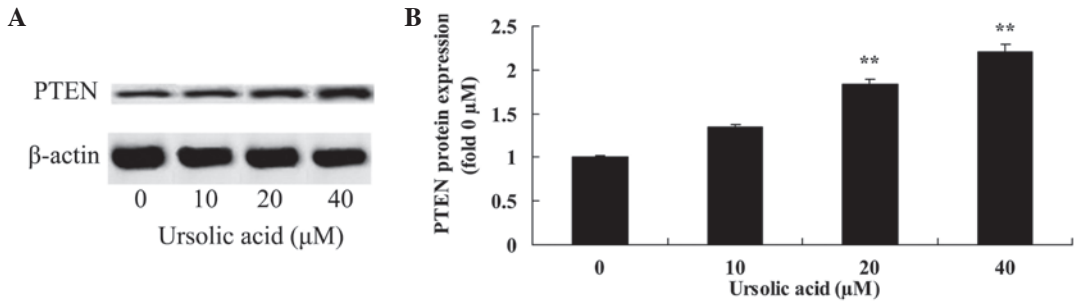

Figure 7. Anticancer effect of ursolic acid treatment on PTEN protein expression in prostate cancer. Anticancer effect of ursolic acid treatment on PTEN protein expression assessed (A) by western blotting assays and (B) quantification of PTEN protein expression in prostate cancer cells. **P<0.01 compared with 0 μM ursolic acid group. PTEN, phosphatase and tensin homolog.

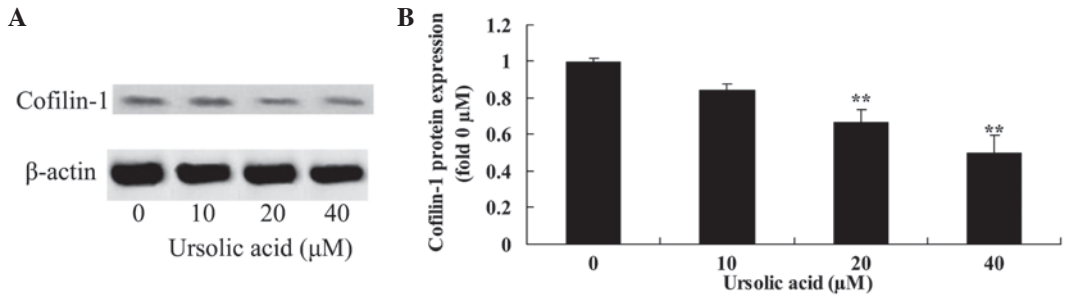

Figure 8. Anticancer effect of ursolic acid treatment on cofilin-1 protein expression in prostate cancer. Anticancer effect of ursolic acid treatment on cofilin-1 protein expression assessed (A) by western blotting assays and (B) quantification of cofilin-1 protein expression in prostate cancer cells. **P<0.01 compared with 0 μM ursolic acid group.

ROCK protein expression in DU145 cells, compared with treatment with 0 μM of ursolic acid (Fig. 6).

Anticancer effect of ursolic acid treatment on PTEN protein expression in prostate cancer cells. To investigate the underlying mechanism of ursolic acid action on prostate cancer cells, PTEN protein expression was measured using western blotting. Following 48 h of ursolic acid (20 and 40 μM) treatment, PTEN protein expression was significantly enhanced in DU145 cells, compared with treatment with 0 μM of ursolic acid (Fig. 7).

Anticancer effect of ursolic acid treatment on cofilin-1 protein expression in prostate cancer cells. To investigate the underlying mechanism of ursolic acid action on prostate cancer cells, cofilin-1 protein expression was measured using western blotting. The results of the present study demonstrated that treatment with ursolic acid (20 and 40 μM) significantly reduced cofilin-1 protein expression in DU145 cells, compared with treatment with 0 μM of ursolic acid (Fig. 8).

Discussion

Prostate carcinoma is common among elderly men and its morbidity in European and American developed countries is high, with up to 650,000 new cases each year (1). In 2008, there were 900,000 new cases of prostate carcinoma globally; therefore, prostate cancer has become the second most serious cancer in terms of its threat to men's health (21). The morbidity of prostate cancer is lower in East Asia; however, due to the aging population, dietary structure alterations and improvements in diagnosis, the morbidity is also rising in countries

in this region (2). The present study observed that ursolic acid significantly suppressed cell growth, and induced apoptosis and caspase-3 and caspase-9 activity in DU145 cells. Previous studies have revealed that ursolic acid is able to suppress cell proliferation and induce apoptosis of APC-mutated colon cancer cells (22), U937 cells (23) and colon cancer-initiating cells (24).

A current focus of cancer research concerns the development of medicines to promote cell apoptosis. Cytochrome *c* is an essential component of the respiratory chain and has a significant role in the oxidation and reduction of cells (25). Utilized as a cell respiration-activating enzyme in the clinic, cytochrome *c* is an ancillary drug used to treat cancer (26). Furthermore, cytochrome *c* additionally has a significant role in apoptosis (27). A previous study reported the extraction of 3 substances associated with cell apoptosis, including cytochrome *c* (28). It was observed that cells in adrenal cortex tumors of mice underwent apoptosis following treatment with cytochrome *c* (27). In the present study, ursolic acid significantly activated cytochrome *c* protein expression in the cytoplasm and suppressed cytochrome *c* protein expression in mitochondria of DU145 cells. Achiwa *et al* (29) suggested that ursolic acid induces apoptosis in SNG-II endometrial cancer cells through reduction of mitochondrial cytochrome *c* release. Li *et al* (25) reported that ursolic acid induced apoptosis of SGC-7901 gastric cancer cells through cytochrome *c*.

Prostate carcinoma cells may express RhoA protein, and the expression of RhoA protein in prostate carcinoma is higher than that in benign prostatic hyperplasia (BPH) (30). Therefore it has been speculated that the formation of prostate carcinoma may be associated with overexpression of RhoA (30,31). The present study observed that prostate cells expressed ROCK/PTEN protein, and the expression of ROCK/PTEN protein in prostate carcinoma is also higher than that in BPH. Furthermore, the expression level of RhoA and ROCK/PTEN protein demonstrates significant positive correlation (30,31). These findings indicated that the ROCK/PTEN signal transduction pathway may participate in the occurrence of prostate carcinoma, and the expression level of ROCK/PTEN protein may be regulated and controlled by RhoA (32). The present study observed that ursolic acid significantly downregulated the ROCK/PTEN signal transduction pathway in DU145 cells. Li *et al* (25) reported that ursolic acid induced apoptosis of SGC-7901 gastric cancer cells through suppression of ROCK/PTEN.

Cofilin-1 is a eukaryotic actin-binding protein with a low molecular mass. The cofilin-1 gene is located at llq13 (32). In addition, cofilin-1 has a large amount of biological activities, including participating in cell apoptosis, cytoplasmic division and affecting phalloidin (33). It has been reported that cofilin-1 protein will translocate into the mitochondria from the cytoplasm; subsequently, cytochrome *c* will be released from mitochondria and combine with Apaf-1 to activate caspase-9, so that the caspase cascade will be activated and lead to apoptosis (19). In addition, the corresponding mechanism of action of cofilin-1 may be associated with the promotion of cyclase-associated protein 1 (25). Furthermore, when cofilin-1 protein translocates into the mitochondria from the cytoplasm, it activates cytochrome *c* release and activation of the caspase cascade, indirectly inducing cell apoptosis (25). The results of the present study demonstrated that ursolic acid significantly inhibited cofilin-1 protein expression in DU145 cells. Previous studies have reported that ursolic acid induces apoptosis of SGC-7901 and BGC-823 gastric cancer cells through suppression of mitochondrial translocation of cofilin-1 (25,34).

In conclusion, the results of the present study suggest that ursolic acid suppresses cell growth, induces apoptosis and increases caspase-3 and caspase-9 activity in DU145 cells. Ursolic acid treatment affects cytochrome *c* protein expression in the cytoplasm and mitochondria, leading to suppression of ROCK/PTEN signaling and mitochondrial translocation of cofilin-1 in prostate cancer cells. Therefore, ursolic acid may present a novel treatment strategy for prostate cancer by targeting ROCK/PTEN and mitochondrial translocation of cofilin-1, leading to activation of apoptosis.

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